C048 Molecular Typing and Surveillance of *Staphylococcus aureus* Isolates Using Pulsed-field Gel Electrophoresis and Multilocus Sequence Typing

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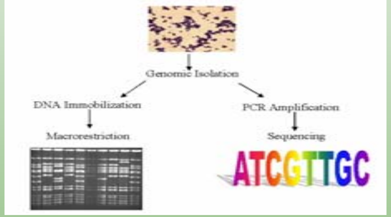
Abstract

S. aureus is one of the leading causes of nosocomial, community, and foodborne infections. The organism has potential use as a bioweapon due to the production of enterotoxin B which is an incapacitating toxin if ingested and a lethal toxin if inhaled. Molecular typing methods are currently being used to detect the spread of antibiotic resistant isolates, to track endemic clones, and will be used to track foodborne outbreaks involving *S. aureus*. In this study two typing techniques, pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) will be compared to determine the advantages and disadvantages of each in the molecular epidemiology of *S. aureus*. It is hypothesized that PFGE will result in a higher level of discrimination among the strains but will result in data that is not easily shared among laboratories, while MLST will result in highly portable data that lacks the discriminatory power of PFGE. *S. aureus* isolates from hospitals in Florida and Washington state and from ATCC were characterized by phenotypic and biochemical methods. Chromosomal DNA fingerprint patterns were generated by macrorestriction with *Sma*I enzyme followed by PFGE. A dendrogram of the resulting patterns was created using the BioNumerics® software. MLST was performed by amplification of seven housekeeping genes for each isolate followed by sequencing. Alleles were assigned using the online MLST database (www.mlst.net). PFGE resulted in 23 pulsotypes whereas MLST resulted in only 4 sequence types (ST) for the same isolates. While a given pulsotype contained a maximum of 6 isolates, 15 isolates were classified as having the same ST type. Isolates that were grouped together by pulsotype were also grouped together by sequence type, but STs often represented more than 1 pulsotype. PFGE provided higher resolution than MLST but the resulting restriction patterns are more difficult to compare than are the numerical data resulting from MLST. The online database allowed easy comparison of MLST data worldwide. Both techniques are expensive and time consuming and a more efficient typing method is needed for outbreak investigations.



Introduction

Staphylococcus aureus is one of the leading causes of nosocomial- and community-acquired infections causing a wide range of disease manifestations including toxin-mediated food poisoning, wound infections, osteomyelitis, and pneumonia (4). The remarkable ability of these organisms to resist most known antibiotic therapies is of particular concern due to the increased morbidity, mortality, cost of treatment, and overall public health burden associated with MRSA infections (3). Recent publications have suggested that community-acquired MRSA is becoming commonplace and that particular *S. aureus* clones feature prominently in epidemics occurring in the United States (6) illustrating the importance of tracking these isolates in the environment. Pulsed-field gel electrophoresis (PFGE) is currently the “gold standard” for tracking *S. aureus* isolates but the technique is labor intensive and results in data that is not easily shared between laboratories. In contrast, multilocus sequence typing (MLST) can be performed in high-throughput laboratories and results in highly portable genomic data. MLST has been used successfully for characterizing methicillin-resistant and methicillin-susceptible isolates (1) and for clonality and evolution studies (2, 5). In the present study, we compared PFGE and MLST for typing 30 community-acquired methicillin-resistant *S. aureus* (CAMRSA) isolates. The isolates were obtained from Florida and Washington states and they represent several disease manifestations.

Materials and Methods

30 *Staphylococcus aureus* isolates were used in this study. Eight isolates are from wounds, 14 from nose/nasal, 5 from blood, 2 from urine, and the remaining isolate is from a bronchial wash. The commonly used *S. aureus* marker isolate NRS77 (NCTC 8325), was used as a control for all pulsed-field gels. All of the isolates are community-acquired *S. aureus* isolates obtained from Harborview and University of Washington Medical Centers in Washington State, and Tampa General Hospital in Tampa, Florida. Community-acquired isolates were those collected within 72 hours of hospitalization, or from outpatient clinics.

Pulsed-field Gel Electrophoresis

Single colony isolates of *S. aureus* cells were grown overnight in TSA broth at 37°C with gentle shaking. Pellets were obtained by centrifugation for 10 minutes at 5100 rpm. Pellets were resuspended in PIV solution (Tris, NaCl). Plugs were prepared by mixing 300 µl of cells with 300 µl of incert agarose. The cell mixture was then aliquoted into plug molds and allowed to cool for 15 minutes at 4°C. Two plugs were prepared in reusable plug molds for each *S. aureus* isolate. Plugs were then placed in 15 ml conical tubes containing 8 ml of EC-lysis solution (Tris, NaCl, detergents, lysozyme, lysostaphin) and incubated for five hours at 37°C with gentle shaking. After five hours, the EC-lysis solution was decanted and replaced with ESP solution (EDTA, sarcosyl, proteinase K) and incubated overnight at 50°C with gentle shaking. The plugs were then washed three times (30-45 minutes for each wash) with TE (Tris, EDTA) at 50°C and stored in fresh TE at 4°C until needed. Plug slices were digested with *Sma*I enzyme overnight at room temperature. Plugs were loaded in 1% SeaKem Gold agarose gels and electrophoresis was performed using the CHEF Mapper (BioRad). Gels were run for 20 hours at 200V using switch times from 5.2- 34.9s. Gels were stained with ethidium bromide and photographed using the GelDoc system. Images were uploaded to the BioNumerics® software package Version 3.0 for analysis.

Multilocus Sequence Typing

The sequences of 7 *S. aureus* housekeeping genes; *arcC, aroE, glp, gmk, pta, tpi*, and *yqi* are required for MLST. Genomic DNA from the 30 *S. aureus* strains was isolated using the MagNA Pure (Roche Diagnostics) system and used as template for amplifying the above genes. The resulting PCR product was purified using the Wizard Promega PCR Prep kit according to the manufacturer’s protocol. Raw sequence data was obtained using a Beckman-Coulter® CEQ 8000 (Fullerton, CA) DNA sequencer following manufacture’s instructions. Sequence data was assembled and analyzed for similarity using the SeqMan program of the DNAStar software package. The resulting alignments were compared to the online *S.aureus* MLST database (www.mlst.net) to assign alleles. Allelic patterns were then used to assign the sequence types.

Results

Pulsed-Field Gel Electrophoresis of *S. aureus* Isolates.

PFGE was performed on the *S. aureus* isolates using *Sma*I enzyme (Figure 1). The resulting patterns were analyzed using the BioNumerics® software package. This analysis allowed the selection for MLST of 30 isolates which posses various PFGE patterns and were from different disease manifestations (Table 1). Included in these were some isolates having the characteristic banding pattern known as USA300, which were used as internal controls for MLST.

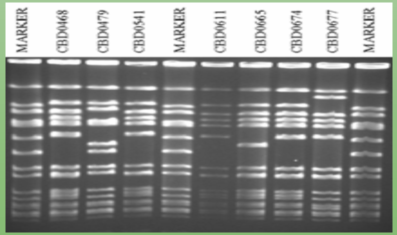


Figure 1. Pulsed-field gel electrophoresis of *Staphylococcus aureus* isolates following macrorestriction using *Sma*I enzyme. The marker strain is the commonly used *S.aureus* isolate NCTC 8325, also digested with *Sma*I.

Amplification of MLST Housekeeping Genes

Genomic DNA isolated using the Magnapure was used as template for amplification of the seven housekeeping genes used for *S. aureus* MLST (*arcC*, *aroE*, *gmk*, *pta*, *glp*, *tpi*, *yqi*) (Figure 2). PCR was performed according to the protocol as given on the MLST website (www.mlst.net) however, PCR for some of the isolates resulted in more than one band for *pta*, and/or *yqi*. PCR annealing conditions were changed for these isolates to eliminate the extra band.

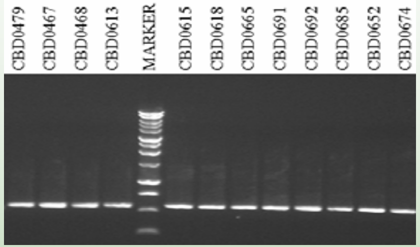


Figure 2. PCR amplification of *glp* gene for MLST before kit purification and sequencing.

Sequencing of *S. aureus* Isolates.

Following DNA amplification, a minimum of four sequences were generated for each housekeeping gene. The resulting forward and reverse sequencing reactions allowed for alignment of the entire gene product using SeqMan. Sequencing reactions ranged from 350 to over 700 bases, but only 450bp was required for assignment of alleles. The resulting alignments were compared to those in the online MLST database and alleles were assigned (Table 1). The resulting allelic profiles were then used to assign sequence types (ST) (Table 1). Only four ST types were assigned among the 30 isolates, the majority of which were ST 8 or ST 5.

Strain	Clinical Source	State	*arcC*	*aroE*	*glpF*	*gmk*	*pta*	*tpi*	*yqi*	ST Type
CBD0467	blood	WA	1	4	1	4	12	1	10	5
CBD0468	wound	WA	3	3	1	1	4	4	3	8
CBD0471	urine	WA	1	4	1	4	12	1	10	5
CBD0472	blood	WA	1	4	1	4	12	1	10	5
CBD0479	abscess	WA	3	3	1	1	4	4	3	8
CBD0482	wound	WA	1	4	1	4	12	1	10	5
CBD0541	wound	WA	3	3	1	1	4	4	3	8
CBD0542	blood	WA	1	4	1	4	12	1	28	105
CBD0611	abscess	FL	3	3	1	1	4	4	3	8
CBD0613	nose	FL	1	4	1	4	12	1	10	5
CBD0614	abscess	FL	1	4	1	4	12	1	10	5
CBD0618	nose	FL	1	4	1	4	12	1	10	5
CBD0633	nose	FL	1	4	1	4	12	1	28	105
CBD0637	nose	FL	1	4	1	4	12	1	10	5
CBD0644	nose	FL	1	4	1	4	12	1	10	5
CBD0652	nose	FL	1	4	1	4	12	1	10	5
CBD0665	nose-throat	FL	3	3	1	1	4	4	3	8
CBD0674	nose	FL	3	3	1	1	4	4	3	8
CBD0677	nasal	FL	3	3	1	1	4	4	3	8
CBD0685	leg	FL	3	3	1	1	4	4	3	8
CBD0691	nose	FL	10	14	8	6	10	3	2	45
CBD0692	nose	FL	3	3	1	1	4	4	3	8
CBD0704	bronchial wash	FL	3	3	1	1	4	4	3	8
CBD0717	nose	FL	1	4	1	4	12	1	10	5
CBD0727A	blood	FL	1	4	1	4	12	1	10	5
CBD0728B	blood	FL	1	4	1	4	12	1	10	5
CBD0734	nose	FL	1	4	1	4	12	1	10	5
CBD0736	nose	FL	1	4	1	4	12	1	10	5
CBD0737	leg wound	FL	3	3	1	1	4	4	3	8
CBD0885	urine	FL	3	3	1	1	4	4	3	8

Table 1. Allele and sequence type assignments for 30 *S. aureus* isolates

Data Analysis – Bionumerics® of *S. aureus* PFGE and MLST Data

*Sma*I-digested *S. aureus* isolates were photographed using a GelDoc system and tiff images were added to a BioNumerics® database. Dendrograms were derived from the unweighted pair group method using arithmetic averages and based on Dice coefficients. The *S. aureus* isolates were grouped into 23 clusters by PFGE as shown in Figure 3. Isolates did not cluster based on either the state in which they were isolated or on their disease manifestations. Isolates with the same pulsotype always had the same MLST sequence type, however isolates with the same sequence type were frequently further differentiated by PFGE (Figure 3) For example, CBD0665 and CBD0677 were both assigned to ST 8 by MLST, but the PFGE patterns differ by >7 bands. The largest cluster of isolates was a match to the recently described USA300 epidemic clone (data not shown) which is also ST 8 by MLST. Sequences of the *glpF* gene were identical for almost all of the isolates and all were assigned to allele 1.

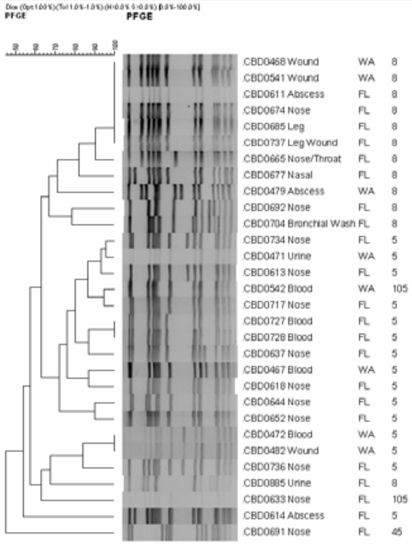


Figure 3. BioNumerics® analysis. Dendrograms were derived from the unweighted pair group method using arithmetic averages and based on Dice coefficients. Band position tolerance and optimization were set at 1.25 and 0.5% respectively.

Discussion

Staphylococcus aureus isolates collected from Florida and Washington state were identified and subtyped by PFGE and MLST. Isolates were selected for the study based on being from different community-acquired disease etiologies including wound, urine, blood, and other manifestations and based on their PFGE patterns. PFGE resulted in 23 pulsotypes as determined by BioNumerics® gel analysis. Isolates were not clustered based on either the state in which they were isolated or on their disease etiology. Multilocus sequence typing analysis resulted in 4 sequence types for the same 30 isolates. Isolates with the same pulsotype always had the same MLST sequence type, however isolates with the same sequence type were frequently further differentiated by PFGE. PFGE is therefore the more discriminatory of the two techniques because it can identify subgroups within each multilocus sequence type. Like PFGE, MLST did not cluster isolates according to state of isolation or disease manifestation. One of the 7 genes used for MLST, *glpF*, was nearly always assigned allele 1 in our study, suggesting that this gene does not add much discriminatory power to the technique. Although housekeeping genes are traditionally used for MLST, the addition of a highly variable gene, such as a virulence determinant, may add discriminatory power to *S. aureus* MLST. The increased spread of *S. aureus* in the community environment, and the remarkable ability of the organism to acquire antibiotic resistance, illustrates the importance of molecular epidemiology for tracking these isolates. To date, there is no readily accessible centralized database for comparing PFGE patterns for *S. aureus* and there is no standard protocol for *S. aureus* PFGE.

Conclusions

- MLST may not be well-suited for tracking community-acquired *S. aureus* as it was unable to distinguish strains that are differentiated by PFGE, and were isolated from geographically distinct locations.
- Regardless of the difficulties involved in performing PFGE for *S. aureus* typing studies, PFGE is the more discriminatory of the two techniques because it can identify subgroups within each multilocus sequence type.
- PFGE patterns from *S. aureus* isolates used in this study did not cluster based on either location of isolation or disease etiology.
- MLST protocols for *S. aureus* may be improved by replacing genes which commonly have the same sequence (*glpF*) with more variable genes such as virulence determinants.

Future Studies

- ➢ Develop protocols for MLST that increase its discriminatory power such as including highly variable genes.
- ➢ Develop standardized protocols for *S. aureus* PFGE and a nationwide database for submitting data.
- ➢ Develop other techniques for tracking virulence determinants in *S. aureus* such as MLVA.
- ➢Determine what characteristics of the epidemic isolates are responsible for their rapid spread worldwide.

References

1. **Enright, M.C., N.P. Day, C.E. Davies, S.J. Peacock, and B.G. Spratt**. 2000. Multilocus sequence typing for characterization of methicillin-resistant and mechicillin-susceptible clonse of *Staphylococcus aureus*. J Clin Microbiol **3**8: 1008-15.
2. **Feil, E. J., and M. C. Enright**. 2004. Analyses of clonality and the evolution of bacterial pathogens. Curr Opin Microbiol **7**:308-13.
3. **Lindsay, J. A., and M. T. Holden**. 2004. *Staphylococcus aureus*: superbug, super genome? Trends Microbiol **12**:378-85.
4. **Lowy, F. D.** 1998. *Staphylococcus aureus* infections. N Engl J Med **339**:520-32.
5. **Robinson, D. A., and M. C. Enright**. 2004. Mutltilocus sequence typing and the evolution of methicillin-resistant *Staphylococcus aureus*. Clin Microbiol Infect **10**:92-7.
6. **Vandenesch, F., T. Naimi, M.C. Enright, G. Lina, G. R. Nimmo, H. Heffernan, M. Liassine, M. Bes, T. Greenland, M.E. Reverdy, and J. Etienne**. 2003. Community-acquired methicillin-resistant *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes: worldwide emergence. Emerg Infect Dis **9**:978-84.

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